Pestic. Sci. 1997, **49**, 353–361

Insecticide Penetration and Symptomology Studies on Larvae of *Diabrotica undecimpunctata howardi* (Barber)

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(Received 14 March 1996; revised version received 11 September 1996; accepted 20 November 1996)

Abstract: The internal and external levels of topically applied soil insecticides, tefluthrin, terbufos and dieldrin, to 3rd-instar larvae of the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Barber), were monitored over 48 h. Symptomology of poisoning with 10 times the dose of the larval 72-h LD_{90} for each chemical was observed over 48 h at various time points after treatment. Terbufos penetrated more rapidly than tefluthrin or dieldrin, although internal levels of tefluthrin and dieldrin had a longer-lasting plateau than terbufos. A 72-h LD_{30} treatment with tefluthrin resulted in faster penetration and also faster disappearance from the insect compared with the 10 × 72-h LD_{90} dose over a 48-h period. Recorded symptoms of poisoning included regurgitation of gut contents, defaecation and writhing and these are suggested to play an important part in voiding of the toxicants at lower (sub-lethal) treatment levels. The sub-lethal effects of tefluthrin are discussed with respect to likely pest behavioural changes, such as anorectic response, after field treatment of a crop.

Key words: terbufos, dieldrin, tefluthrin, penetration, symptomology, insecticide pharmacokinetics, *Diabrotica* sp.

1 INTRODUCTION

Early studies[1] examined the species-specificity of insecticides and their inherent toxicity by experimenting with different routes of entry including by-passing the cuticle, the natural barrier found in insects. Brooks[2] considered that most insecticides pass through the cuticle by passive diffusion across a concentration gradient, resulting in a linear plot of percentage recovery against time. However, this has not always been found to be the case.[3–6] The reasons for deviations from a straight-line response are many, but penetration through successive cuticular layers with varying degrees of permeability to compounds of differing polarity is thought to be an important determinant.[4,5,7–9] The processes of excretion, volatilisation from the cuticle, type of solvent used, rate of metabolism, accumulation in body organs and activation are also influential on the penetration rates of parent insecticides.[5,10–14] Many workers[6,10,11] have measured the levels of parent compound (often radio-

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Pestic. Sci. 0031-613X/97/$17.50

labelled) remaining on the insect cuticle after application. Additionally, internal body extracts, using appropriate solvent extraction techniques have been used to assess penetration rates.[11,15,16] Studies using radioactively labelled insecticides generally provide greater accuracy of total compound partitioning in treated insects.[3,10,15,17,18]

At lethal and sub-lethal doses, toxic effects inducing symptoms of poisoning are seldom fully described in bioassays for insecticide potency. More frequently, such observations are found in studies on pharmacokinetics of insecticide action, although few studies have followed the time course of poisoning and concomitant insecticide levels in the insect.

The current work represents an attempt to describe physiological symptoms and the penetration of selected soil insecticides with time in a larval stage of the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Barber), (Coleoptera: Chrysomelidae). As a major pest in the soils of the maize-growing area of the USA mid-west, the treatment levels were selected to reflect pesticide concentrations likely to be found in the soil following field application.

2 MATERIALS AND METHODS

2.1 Chemicals

In all cases, technical material of the following insecticides was used: dieldrin (1*R*,4*S*,4a*S*,5*R*,6*R*,7*S*,8*S*,8a*R*)-1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-1,4 : 5,8-dimethanonaphthalene), 99% purity, Sigma Chemicals; terbufos (*S-tert*-butylthiomethyl *O*,*O*-diethyl phosphorodithoate), 98·6% purity, American Cyanamid Inc.; tefluthrin (2,3,5,6-tetrafluoro-4-methylbenzyl (*Z*)-(1*RS*)-*cis*-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate), 99% purity, ICI (now Zeneca Agrochemicals Ltd). Stock and test solutions were freshly prepared in Analar® grade acetone on day of use.

2.2 Insects

The laboratory colony of *D. undecimpunctata howardi* was established from a sub-population (ex May and Baker, Essex, UK) originating from a susceptible laboratory-reared stock in the USA corn belt. Detailed laboratory culture methods were derived from a variety of sources and are described elsewhere.[19,20] Larvae were reared in vented plastic containers filled with sedge peat (Sedgemoor Drain, Devon, UK), fed uncontaminated, germinating maize (*Zea mays*, L. hybrid field corn AG10, W. W. Johnson & Son, Boston, Lincs., UK) at 27(±2)°C with a 16-h photophase. For all the bioassays, mid-late 3rd-instar larvae (four to five days into L3 stage) weighing between 12 and 15 mg each were used.

2.3 Symptomology studies

The treatments applied were 10 times the estimated 72-h LD_{90} dose (all compounds) and the 72-h LD_{30} dose for tefluthrin, based on topical application data of 3rd-instar (L3) *D. undecimpunctata howardi*.[20] Tefluthrin was analysed at the lower dose following preliminary studies on the dose-induced behavioural changes due to topical, deposit and soil application of the insecticide.[20] Larvae were placed on glass Petri dishes and 0·2 µl (uncalibrated volume) of test solution was applied to the dorsal thorax (time zero) using a Hamilton Microlab P dispenser (Hamilton GB Ltd, Dundee, UK). Three replicates of 10 larvae were treated for each compound. Once treated, each group of 10 larvae was placed in a covered glass Petri dish (9 cm dia.) with a water source to maintain a constant humidity and the dishes were kept at 25(±2)°C with a 16-h photophase. Larvae were given germinating maize as a food supply. The behaviour and responsiveness of each group of larvae was observed for 1–2 min at 0·5, 1, 2, 4, 16, 24 and 48 h after treatment. Acetone-treated and untreated insects were examined as controls.

Symptoms were classified by the behavioural changes following treatment relative to controls. Behaviour of treated, moribund insects was assessed using a combination of the methods of past workers. These suggested that curling and writhing activity,[21] uncoordinated, erratic head and thoracic movements, inability to crawl away from tactile stimulation,[22] rolling and partial paralysis[23,24] were typical insecticide-induced symptoms. In addition, the strength of head capsule retraction after stimulation by a seeker was used to gauge responsiveness. The behavioural studies tests were repeated twice to ensure standardisation of response.

2.4 Penetration experiments

In an identical experimental design, the compounds used in the behavioural study (Section 2.3) were applied topically at the same doses. Each insect replicate group was then placed in a glass vial (25 ml, 5·5 × 2·5 cm) with a damp cotton wool plug in the lid. The lids were loosely fitted and the vials laid horizontally on the bench. The vials were covered to reduce light-induced movements. Insects were taken at time zero and at 0·5,

TABLE 1
Gas Chromatograph Conditions for Insecticide Analysis

Chromatograph	*Terbufos*	*Dieldrin*	*Tefluthrin*	*Tefluthrin* (LD_{30})
Detector	PID	ECD	ECD	ECD
Detector temperature (°C)	230	250	250	300
Injection temperature (°C)	230	230	230	250
Column temperature (for external body or vial wash) (°C)	170	240	170	175
Column temperature (for internal body extracts) (°C)	150	240	170	175
Stationary phase	[a]	[a]	[a]	[b]
Carrier gas, helium (ml min^{-1})	20	20	20	20
Make up gas, nitrogen (ml min^{-1})	30	30	30	60
Internal standard	212H87[c]	Cyhalothrin	110H90[c]	Dieldrin
Extraction efficiency (%)	91	93	81	98
Retention time (min)	5·4	2·7	6·4	2·6

[a] Durabond DB5 Megabore (phenylmethylpolysiloxane) stationary phase, 0·53 × 10 m BF71303 packing.
[b] 2% OV-101 silicone stationary phase, Gaschrom Q 80/100 mesh packing 4 mm × 1·5 m.
[c] In-house sulfur-containing organic internal standards (technical material of suitable retention time, composition and physical properties).

1, 2, 4, 16, 24 and 48 h for body washes and extraction procedures (three vials per time period).

2.4.1 Body washes

To determine the amount of unpenetrated parent compound, replicate groups of larvae were placed in acetone (2 ml) in glass vials (4 × 1·2 cm) and gently shaken for 30 s. The larvae were then carefully removed with fine tweezers and hexane (0·5 ml) slowly pipetted over them into the acetone rinse vial. The larvae were transferred into a clean glass container at 4°C for subsequent internal extraction (Section 2.4.2). At this point, 5 μl of a 0·1 mg ml^{-1} solution of an internal standard (see Table 1) was added to each acetone rinse vial. The vials were then gently shaken for another 30 s. The solvent was then evaporated down to 50–75 μl under nitrogen and transferred to tapered vials and sealed prior to analysis by gas chromatography (GC). Samples were stored in the dark at 4°C. Glass vial rinses that held the larvae during the experiment were treated in a similar manner to evaluate rub-off, regurgitation and excreted parent insecticide.

2.4.2 Extraction

The acetone-washed larvae (see Section 2.4.1) were homogenised in glass homogenisers (20 ml) with acetonitrile (0·5 ml) for 1 min. Distilled water (0·5 ml) was added and the mixture re-homogenised for 1 min. At this stage, 5 μl of 0·1 mg ml^{-1} internal standard (see Table 1) was added and the material homogenised for a further 30 s. The homogenate was then transferred to 2-ml glass vials (3·5 × 1 cm). Hexane (0·5 ml) was used to rinse the homogenisers and added to the vials. The aqueous suspension in the vials was then extracted with the hexane by gently shaking for 20–30 s. The vials were then placed in a microcentrifuge (Beckman microfuge E, High Wycombe, UK) and centrifuged for 10 s at 6000–8000 rev min^{-1} and the supernatant hexane layer partitioned. The hexane layer was removed carefully and placed into a clean glass vial. The extraction centrifugation procedure was repeated twice and the samples pooled. The vial contents were then evaporated under nitrogen to 0·5 ml, sealed and stored in the dark at 4°C ready for analysis by GC (Section 2.5).

The extraction efficiency of the above procedures was determined by application of the test compounds to larvae followed by immediate extraction and the addition of internal standards (see Table 1) to obtain a percentage efficiency measure of the initial application. To calibrate the test doses, they were also applied to glass coverslips in 10, 8, 6, 4 and 2, 0·2-μl drops per coverslip for each compound (representing 10, 8, 6, 4 and 2 larval applications). The coverslips were placed in vials and acetone (2 ml), hexane (0·5 ml) and internal standard (5 μl; see Table 1) added. The volumes were adjusted as before to 50–75 μl. These calibration tests were also used to obtain optimum sensitivity and GC conditions (Section 2.5).

2.5 Gas chromatography

Insecticides were analysed using a Hewlett Packard 5890 Series II Gas Chromatograph linked to a Hewlett Packard 7673 Controller and HP 3396A Integrator. For

TABLE 2
Feeding and Associated Gut System Symptoms Exhibited by Larvae of *Diabrotica undecimpunctata howardi* Induced by Topical Applications of Insecticides. (10 × 72-h LD_{90} dose): Observed Behaviour or Effect[a]

		Time after application (h)[b]						
Behaviour	*Treatment*[c]	*0·5*	*1*	*2*	*4*	*16*	*24*	*48*
Feeding	A							
	B							
	C							
	D						+	+++
Regurgitation	A	+++	+	+	+	+		
	B			+	+			
	C	+++	+++	+				
	D	+		+				
Swollen abdomen	A	+	+	++	++	++	+++	+++
	B				+	++	++	++
	C	++	+	+	+	+	+	+
	D	+	+					
Anal/hind gut extension	A	+	+	+	+	+	+	
	B				+			
	C		+	+	+			
	D	+	+					

[a] + = observed behaviour/effect in <30% of insects, ++ = observed behaviour/effect in 30–60% of insects, +++ = observed behaviour/effect in >60% of insects.
[b] Three replicates of 10 insects.
[c] A = terbufos B = dieldrin C = tefluthrin (high dose) D = tefluthrin (low dose).

the sulfur-containing terbufos, a photo-ionisation detector (PID) was used; for the halogenated dieldrin and tefluthrin, an electron capture detector (ECD) was used. The GC conditions and settings are shown in Table 1. Using the integrator, peak areas were used to obtain the compound : internal standard ratio plotted against percentage compound applied to coverslips (10 × 0·2-μl drops = 100%) to obtain a calibration curve. Penetration and cuticular loss rates were calculated by the log percentage of insecticide extract or body wash plotted over time to obtain a straight line. The slope of this provided a rate constant (% min^{-1}).[17]

3 RESULTS

3.1 Symptomology of poisoning

All symptoms described are those that normally classify these insects as moribund.[23] Dead insects were those that did not respond to any stimulation and were obviously dead (flaccid, discoloured, putrefying bodies).

Terbufos produced regurgitation of gut contents within 30 min of application to the larvae (Table 2) and induced a convex bending of the body (ventro-dorsally). Abdominal swelling and curling was quite pronounced, the anus was extended and the hindgut was often extruded. Rapid defaecation was common from the time of application. The anterior portion of the body exhibited writhing and twitching of the prolegs (Table 3). Initial mortalities occurred at 16 h; the survivors remained curled and bloated. No feeding was observed at any stage and response to stimulation was lost after the initial 30 min.

Gut distension, body-curling and anal extension occurred after 4 h following dieldrin treatment (Tables 2 and 3). Dieldrin also produced lethargic larval movements and only a slight response to head capsule stimulation (Table 3). Feeding ceased immediately. There was little change in activity until 2 h post-treatment when writhing and defaecation was evident.

The tefluthrin treatment resulted in very high regurgitation activity in the first hour following treatment, with writhing and abdominal distension (Tables 2 and 3). No feeding occurred at any stage of the study. The larvae became elongated and there was obvious anal extension. No response to stimulation was observed. Convulsions and writhing movement were very strong throughout the time course. Body curling began after 2 h but, unlike terbufos and dieldrin-treated larvae, swollen abdomens were not strongly characteristic of tefluthrin poisoning. At the lower tefluthrin dose, larvae showed the least response of all treatments. Regurgitation and abdominal swelling were slight and short-lived and the feeding resumed after 24 h in some larvae and in all survivors by 48 h (Table 2). Apart from the anorectic reaction (common to all treatments), the main

TABLE 3
Neuromuscular and Associated Symptoms Exhibited by Larvae of *Diabrotica undecimpunctata howardi* Induced by Topical Applications of Insecticides. (10 × 72-h LD_{90} dose): Observed Effect[a]

		Time after application (h)[b]						
Behaviour	*Treatment*[c]	*0·5*	*1*	*2*	*4*	*16*	*24*	*48*
Writhing	A	+++	+			++	+	
	B			++	+	++	++	+++
	C	++	++	+	++	+++	+++	++
	D	+	+++	+++	+++	++	+	
Uncoordinated motion	A							
	B			+				
	C				++	+		
	D							
Lethargy	A							
	B		+	++				
	C							
	D							
Curling	A	+	+	++	+++	+++	+++	+++
	B		+	+++	+++	++	+	
	C			++	++	+++	+++	
	D						+	
Paralysis (abdomen)	A	+	++	++	+			
	B							
	C							
	D			+	+	+		
Responsiveness	A	++						
	B	++	+	+			+	
	C							
	D					+	++	+++
Mortality (%)	A	0	0	0	0	20	40	100
	B	0	0	0	0	10	10	40[d]
	C	0	0	0	0	0	10	40[d]
	D	0	0	0	0	0	0	30[e]

[a] + = observed behaviour/effect in <30% of insects, ++ = observed behaviour/effect in 30–60% of insects, +++ = observed behaviour/effect in >60% of insects.
[b] Three replicates of 10 insects.
[c] A = terbufos B = dieldrin C = tefluthrin (high dose) D = tefluthrin (low dose).
[d] 100% mortality at 72 h.
[e] No further mortality at 72 h.

symptoms observed were writhing and convulsions. After 48 h, a full response to stimulation returned (Table 3).

3.2 Penetration studies

Terbufos exhibited the most rapid loss from larval cuticle of all the applied insecticides, with no parent compound detected after 4 h (Fig. 1). Glass vial rinse levels of terbufos were similar over the 4-h period. The internal terbufos body levels only dropped considerably after 24 h (Fig. 2). Overall, the total recovery of terbufos was relatively poor (max. 63% total recovery after 30 min). Losses of terbufos may have resulted from evaporation from the cuticle and/or the container following rub-off. This effect has been reported before with volatile carbamates[24] and with other organophosphates.[25]

There was a marked difference in the penetration rates of dieldrin and tefluthrin compared with terbufos. With both of the halogenated insecticides, the loss from the cuticle was much slower, particularly with tefluthrin (Fig. 1). This corresponded with a slower appearance of the parent compound inside the body compared with terbufos. Internal levels of parent dieldrin were the highest of all insecticides. Poor total recovery of dieldrin was observed (70% after 30 min).

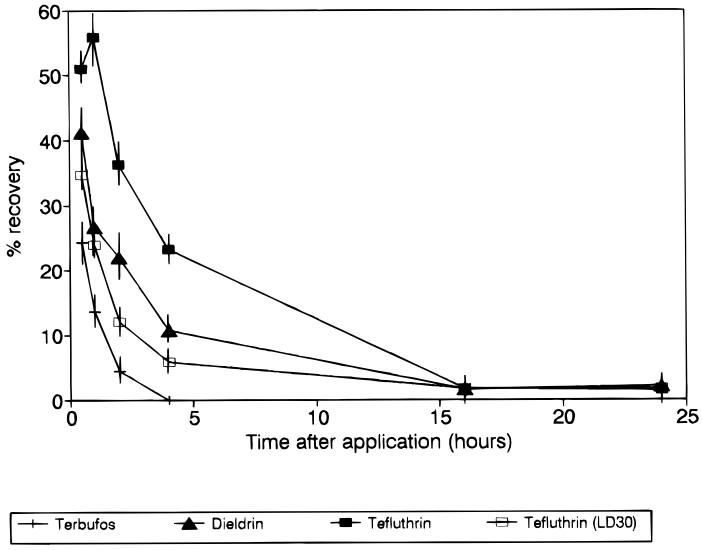

Fig. 1. Comparative recovery of insecticides from external body washes following topical application of 10 × 72-h LD_{90} dose (unless otherwise indicated) to 3rd instar *Diabrotica undecimpuncata howardi* larvae. Bars equal SEM.

The external levels of tefluthrin at the lower dose dropped more rapidly than with the high dose (Fig. 1). Similarly, the internal body levels of tefluthrin rose more sharply and reached a sustained level for longer at the lower dose than with the high dose over the first 16 h (Fig. 2). There was a rise in the tefluthrin levels found in the glass vial rinses towards the later stages of the study. Tefluthrin recoveries at the higher and lower doses were 83 and 90%, respectively, after 30 min.

4 DISCUSSION

Larval regurgitation coupled with writhing activity may have led to the relatively high levels of terbufos found in the glass vial rinses. Similar losses were also observed by Hsin[22] using bendiocarb and isofenphos against *D. undecimpunctata howardi* larvae. Loss of toxicants by rub-off and vomiting has been reported for other insects showing similar regurgitant and writhing behavioural reflexes: *Musca domestica* L.,[16] *Spodoptera littoralis* (Boisduval)[26] and *Popillia japonica* Newman.[27]

The observed decline in the internal level of terbufos was probably not due to excretion of the parent compound, as body and container rinses showed no evidence of terbufos at these times. The possible formation of an unextractable conjugation product[28] of terbufos within the insect body may explain the poor recovery of parent compound. The metabolic transformation of terbufos to the toxic sulfoxide and sulfone derivatives is

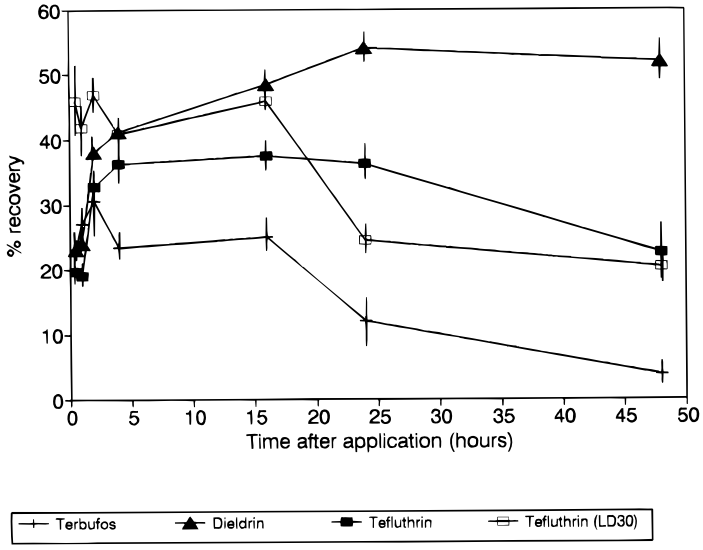

Fig. 2. Comparative recovery of insecticides from internal body extracts following topical application of 10 × 72-h LD_{90} dose (unless otherwise indicated) to 3rd instar *Diabrotica undecimpuncata howardi* larvae. Bars equal SEM.

also known in insects.[29] In contrast, Chio and Metcalf[30] found that, in adults of *D. longicornis* Say, *D. undecimpunctata howardi*, *D. virgifera* Le Conte and *Acalymma vittata* (F.), unchanged terbufos was high throughout the experimental period of 24 h indicating the inherent toxicity of the parent compound and the low detoxification rate by the imagos of these species. In the present study, the GC conditions and even the sample preparations may not have been ideal for detection of metabolites. The appearance of unidentified peaks may or may not have been evidence of metabolites.

In dead insects, penetration and internal levels of insecticides tend to reach a plateau fairly rapidly and do not change significantly with time,[2] indicative of the abatement of enzyme activity and excretory processes. However, Hsin[22] found that penetration, metabolism and excretion by dying insects was slower than in living ones although the definition of dead or moribund was not stated. In the present work, a rapid rise in internal levels of insecticides (moribund or dead) was only observed with dieldrin and both tefluthrin doses, whereas terbufos showed a slower rise and steady decline in internal levels. The lipophilic nature of tefluthrin and dieldrin (indicated by the logP and low solubility values, Table 4) implies strong retention by tissues or target sites and hence little or no metabolic degradation.

The penetration data and curves for both dieldrin and tefluthrin (Figs 1 and 2) were very similar. Total recovery of dieldrin was low at 70% and, unlike terbufos, this cannot be related to its volatility. It is quite likely that dieldrin and tefluthrin, by virtue of their lipophilicity (Table 4), had a large proportion of the parent compound locked in the cuticular layers of the insect.[6] Lipophilic compounds, such as many of the organochlorines,[6,11,13] some pyrethroids[26] and avermectins,[18] are well known adherents to epicuticular waxes in these layers. However, Treherne[7] suggested that epicuticular waxes had less influence than first thought, although his work was on the relatively thicker cuticle of *Schistocerca gregaria* (Forsk.) treated with ureas of varying solubility.

Compared with terbufos, dieldrin and tefluthrin showed a slower rise in internal levels. The observations on dieldrin agreed with the work of Sun[5] who suggested that organochlorines generally penetrated more slowly than organophosphates. Dieldrin and tefluthrin also remained on the cuticle at higher levels for longer than terbufos. Tefluthrin and dieldrin also have lower vapour pressures than terbufos and this may influence the strength of their adherence to the epicuticular waxes. The estimated penetration rate constants from the present study (Table 4) supported previous observations of higher values for organophosphates than for chlorinated insecticides.[5]

No regurgitation was observed following dieldrin treatment. However, regurgitation and writhing of larvae 30 min after treatment with the higher dose of tefluthrin was correlated with an increased level of tefluthrin in the bottle and body washes. This purging activity has also been recorded for other pyrethroids, especially with *S. littoralis*.[16,26]

The lower-dose treatment of tefluthrin (Fig. 1) was in marked contrast to the higher, lethal dose. In the former, writhing was common but much less regurgitation was observed. A slight increase of compound in the bottle washes was evident and, although this was lower than with the higher, lethal dose, some excretion or regurgitation did occur. As part of a detoxification mechanism of low treatment levels of an insecticide, regurgitation or purging of the gut contents of the toxicant may be quite an effective strategy, considering the eventual recovery of the sub-lethally-treated tefluthrin insects. Brealey,[16] for instance, found the regurgitated gut contents from pyrethroid-treated *S. littoralis* larvae to be 85% parent compound.

Penetration of tefluthrin into the body was more rapid at the lower dose and corresponded with a very rapid rise in internal levels which eventually reached a higher level than that of the higher, lethal dose. After 16 h, the toxic symptoms of surviving 3rd-instar *D.*

TABLE 4
Physical Parameters and Rate Constants of the Insecticides under Study

Compound[a]	*Vapour pressure*[b] (*mPa*)	*logP*	*Solubility in water* (*mg litre*$^{-1}$)	*Penetration rate constant at 25°C* (% *min*$^{-1}$)	*Cuticular loss rate constant at 25°C* (% *min*$^{-1}$)
Terbufos	34·6 (25°C)	4·5[b]	4·5(20°C)[b]	0·0098	0·0450
Dieldrin	0·40 (20°C)	5·4[c]	0·19[d]	0·0130	0·0220
Tefluthrin	8 (20°C)	6·5[e]	0·02 (20°C)[b]	0·0110	0·0078
Tefluthrin (LD_{30})	—	—	—	0·0620	0·0280

[a] 10 × 72-h LD_{90} dose unless stated.
[b] After Tomlin.[32]
[c] After De Bruijn *et al.*[33]
[d] After Worthing and Walker.[34]
[e] After McDonald *et al.*[35]

undecimpunctata howardi larvae from the lower dose diminished compared with those of the high dose treatments and were more like the control groups, although the moribund insects (30%) eventually died after 72 h. Complete recovery of the remaining insects occurred by the end of the behavioural study at 96 h. Interestingly, the internal levels of tefluthrin-treated larvae were not significantly different ($P > 0{\cdot}05$) after 48 h between the low and high dose treatments although recovery took place in the former.

It is likely that the majority of the high internal body concentration levels of parent compound were due to the pooled results which included the dying insects in each of the lethal treatments. Voiding of toxicants from dead bodies would not occur and with inclusion of these insects, the measured levels were possibly higher (and additive) than if these insects had been removed on death. This was suggested from the proposition of differential excretion and metabolism by moribund insects.[2,22]

The present results for tefluthrin (Fig. 2) compare well with observations on the cutworm *Agriotes ipsilon* (Hufn.),[31] where higher doses of carbaryl, vamidothion and fenvalerate resulted in lower penetration rates than lower (sub-lethal) doses. However, a study on the effects of organophosphates on houseflies found that lower doses increased the time for internal distribution of the pesticides.[15]

The current work has shown that single topical applications of a low, relatively sub-lethal dose of tefluthrin caused similar initial symptoms to a lethal dose, albeit of lower intensity, the most important symptom being reduced feeding of the larvae and the possibility of voiding of toxicants by regurgitation. A longer exposure to a low-level, sub-lethal toxicant could produce a profound effect on *D. undecimpunctata howardi* larval behaviour and subsequent feeding activity. This would provide further information on the plant protection profile and activity of a compound at sub-lethal levels, in addition to pure kill at suggested lethal field-rate recommendation levels in the field.

ACKNOWLEDGEMENTS

We are indebted to Rory Brett and John Heekin for their time and efforts during the preparation and optimisation of conditions for the GC analysis. We would like to thank Lisa Johnson for help in the preparation of the manuscript and Dr A. J. Adams for his review of the drafts. Additionally, we would like to thank Dr S. N. Irving for his support of this work.

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